United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	The effects of toxicants with neuroendocrine sites of action on ovulation in the adult female rat.
LAPR Number:	18-09-003
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6/RTP/USEPA/US
Date Originated:	07/28/2015
LAPR Expiration Date:	09/30/2018
Agenda Date:	09/09/2015
Date Approved:	10/27/2015
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	10/27/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	10/27/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
The effects of toxicants with neuroendocrine sites of action on ovulation in the adult female rat.

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
Research Program: Chemical Safety for Sustainability (CSS)
Project: Adverse Outcome Pathway Discovery and Development
Task Title: Development of AOPs for Reproduction in Vertebrates
Task Number: 1.3b

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/TAD/Exemption Exemption Exemption/2013-001-r000

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	**Exemption 6**	TAD	MD
	Lotus Notes Address	***Branch***	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US	ETB	

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	**Exemption 6**		MD
	Lotus Notes Address	***Branch***	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US		

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific

persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

The main objective of this study is to identify adverse outcome pathways (AOPs) that lead to perturbed ovulation in the female rat. AOPs are flow charts designed to link chemical exposures with adverse biological outcomes to disease states or developmental abnormalities. An AOP starts with the disruption of a cellular process by a chemical called a molecular initiating event (MIE). An MIE leads to a cascade of biological changes that ultimately leads to an adverse condition or state.

Ovulation is a key event in reproduction necessary for the release of an egg prior to the arrival of sperm. This event is caused by the surge of a hormone called Luteinizing Hormone (LH) released from the anterior pituitary. The surge of LH is dependent upon the release of gonadotropin releasing hormone (GnRH) from neurons in the Hypothalamus. The goal of our lab through this study is to identify chemicals that alter neuroendocrine regulation of these processes and disrupt hypothalamic control of ovulation. Chemicals in question may inhibit ovulation by altering steroidogenesis, interrupting neurotransmitter synthesis, or by disrupting neurotransmitter binding sites.

Ultimately, data collected from in vivo experiments will be used to create in vitro assays that are predictive of rodent model AOPs. Therefore, in these initial in vivo studies we will examine the effects of compounds that may affect ovulation by several potential mechanisms: blocking synthesis of hormones necessary for ovulation (Celecoxib and Flurbiprofen), inhibiting enzyme activity necessary for production of the neurotransmitters required for ovulation (sodium dimethyldithiocarbamate), or by interferring with receptors in the hypothalamus that regulate ovulation (Difenzoquat). Dose response studies of these compounds will provide valuable information on the ability of the chemicals to disrupt estrous cyclicity and ovulation. Once these in vivo effects are established we can use that information to design and test in vitro high-throughput systems and establish testing strategies for evaluating other compounds that may perturb ovulation in a similar manner. Thus, the knowledge acquired through this project will be beneficial to future work in which identification of harmful compunds that affect ovulation will be recognized through in vitro processes and reduce the overall need for in vivo animal models.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of animals is necessary for the development and validation of AOPs as only animal models can provide the complex interactions of the neuroendocrine system that is exhibited by an intact animal. Results from in vitro experiments evaluating the contribution of various compounds to AOPs will inevitably be compared to the results obtained from these current in vivo experiments performed in intact animals.

b. Justify the species requested:

Rats and humans exhibit a high degree of conservation in regards to neuroendocrine regulation of ovulation including the pathways and targets of interest in this study. Furthermore, in the field of endocrine disruption rats have been utilized as a common model species. Therefore, there is an abundant amount of literature from which to gain insight, plan appropriate and accurate studies, and from which to make fair and accurate comparisons to the current work.

3. How was it determined that this study is not unnecessary duplication?

Endnote version 17 was used to search the PubMed database using multiple terms in conjunction with each other. The search terms "celecoxib&ovulation, celecoxib&LH surge, and celecoxib & female reproduction" were searched. Similar search terms and combinations were used for flurbiprofen and difenzoquat. These searches revealed that our approach to investigating the effect of Celecoxib (a COX2 specific inhibitor), Flurbiprofen (a Cox1 specific inhibitor), and Difenzoquat (alpha 2 adrenergic receptor agonist) using the rat as a model species is novel.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Mature female rats exhibiting normal 4-day estrous cycles that are approximately 60 days old and 250

grams body weight will be dosed by oral gavage. A negative control group will be dosed with the dosing vehicle pharmaceutical grade physiological saline (0.9%) or methyl cellulose (1%) depending upon the solubility of the compound. A positive control group will be dosed with dimethyl dithiocarbamate (DMDC) which is known to inhibit the LH surge. Each experimental treatment group will be dosed with a novel compound that has exhibited the potential to alter neuroendocrine control of the LH surge and disrupt ovulation. Treatment group 1 will be dosed with Celecoxib, a COX2 specific inhibitor. Treatment group 2 will be dosed with Flurbiprofen, a COX1 specific inhibitor. Treatment group 3 will be dosed with Difenzoquat, an alpha2 adrenergic receptor agonist.

Dosing of animals will occur at 1300 hrs on the day of Proestrus. The low, middle, and high dose for each test chemical will be set at approximately 1%, 5%, and 10% of the LD50, respectively. Half of the animals will be killed by decapitation 24 hours later on the day of Estrus. The other half will be killed by decapitation 48 hours after dosing. The oviducts from each animal will be recovered and flushed to determine the number of oocytes ovulated. Blood serum will also be collected to examine each animal's respective hormone profile.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

Based on our previous experience with studies of LH surge and ovulation inhibition in female rats, we propose that we will need 16 females per treatment for each dosage to obtain the number of animals necessary to achieve statistical significance. For each chemical group, we estimate that we would need 80 animals.

	(Saline Methyl Cellulose) Control (-)	DMDC Control (+)	Test Chemical (Celecoxib, Flurbiprofen, or Difenzoquat) Low	Mid	High
24 hr	8	8	8	8	8
48 hr	8	8	8	8	8

If 3 chemicals were tested, then a total of 240 animals would be needed. However, based on our previous historical data on this strain of rat, we expect approximately 60% of the females to exhibit a 4-day estrous cycle. For this reason, we will need to order 400 adult females in order to obtain the 240 necessary to ensure that we can complete the study with the specified number of females/group. Females that do not exhibit a 4-day estrous cycle will either be transferred to another LAPR or euthanized at the end of the study.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	400	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

Experimental chemicals will be dissolved in pharmaceutical grade physiological saline (0.9%) or methyl cellulose (1%). Either saline or methyl cellulose will be used as the vehicle dependent upon the chemicals solubility. Saline was chosen over deionized water as physiological saline more closely resembles the internal environment of a life animal as it relates to tonicity. Dosing volumes will be based on rat body weight. Dosing solutions for oral gavage will be prepared so that the animal receives 5mL per kilogram of body weight. For example, if a 250g (i.e., 0.25kg) rat were to be dosed with sodium dimethyldithiocarbamate at 6.25mg/kg, then 6.25mg of sodium

dimethyldithiocarbamate would be dissolved in 5mL of saline. The rat would then be dosed with 1.25mL of 6.25mg sodium dimethyldithiocarbamate/5mL saline solution.

For this study, dosing will include:

Test Agent	Dose	Target
Sodium Dimethyldithiocarbamate	25 mg/kg	Inhibitor of Dopamine beta-hydroxylase
Flurbiprofen	0 - 12 mg/kg	Inhibitor of Cycloxygenase 1
Celecoxib	0 - 200mg/kg	Inhibitor of Cycloxygenase 2
Difenzoquat	0 - 25 mg/kg	Alpha2 Adrenergic Receptor agonist

b. Survival Blood Collections (method, volume, frequency):

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Vaginal smears obtained via vaginal lavage will be utilized to confirm estrous cyclicity for the duration of the experiment. Vaginal lavage is performed by gently flushing the vaginal cavity with approximately 0.5ml of water using a glass eye-dropper.
d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:
Adult females will be housed two per cage with one of them receiving an ear punch to distinguish between the two.

Animals will be monitored daily and body weights will be measured throughout the treatment timeframe by the technical staff of Exemption 6Exemption 6Exemption 6Exemption 6 Exemption 6 technical staff will closely monitor for signs of any systemic toxicity including body weight loss or lack of appetite (see section B8).

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

c. Testing methods:

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint

is highly discouraged:

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

◯ Yes ◯ No

f. Identify any surgical procedures performed at other institutions or by vendors:

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Symptoms of toxicity could inlcude lethargy, rough hair coat, body weight loss >10%, or deteriorating body condition. However, because the experimental design calls for only one dose per animal well below the LD50 of a given chemical, we do not expect to see any overt effects of the treatment.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

If any animals appear to be sick by displaying signs of systemic toxicity, appear to have increased stress, and/or not eating, then they will be promptly removed from the study and immediately euthanized. Signs of systemic toxicity may include weight loss, lethargy, and rough hair coat.

The attending veterinarian will be consulted when appropriate to determine the appropriate course of action.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 400

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 400

2. Species (limited to one per LAPR): rat(s)

3. Strain: Wistar rats

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

4. Sources of animals:

Charles River Laboratories

5. Provide room numbers where various procedures will be performed on animals:

Exemption 6

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

N/A

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

N/A

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

We may need assistance from animal contract staff with dosing, body weight measurements, and smears via vaginal lavage.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

We request that polycarbonate cages with heat treated pine shavings be used. Our study will require that only pine shavings be used to prevent endocrine disruptor exposure from alternative bedding material or enrichments.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles,

recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

Chemical	Maximum Dosage	Oral rat LD50
Sodium Dimethyldithiocarbamate	25mg/kg/day	1000mg/kg
Flurbiprofen	12mg/kg/day	117mg/kg
Celecoxib	200mg/kg/day	>2000mg/kg
Difenzoquat	25mg/kg/day	270mg/kg

Vehicles will be saline and methylcellulose solutions.

Celecoxib: HSRP 805 The evaluation of chemicals on the hypothalamic-pituitary-gonadal (HPG) axis and development.
None of the other chemicals listed require an HSRP. Gloves, eye protection, and lab coat will be warn when handling.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.
Animals will be dosed with pharmaceutical grade Sodium Dimethyldithiocarbamate, Flurbiprofen, or Celecoxib. Pharmaceutical grade Difenzoquat is unavailable.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.
Personal protective equipment will be donned including gloves, lab coat, and protective eye wear.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Develop study and assist with organization or any animal handling	Over 25 years of animal handling and performing research studies for endocrine studies. Has had all NHEERL-required

		necessary	training.
Exemption 6	Post-Doc	Develop study and assist with organization or any animal handling necessary. Prepare doses, monitor animals, and perform necropsy.	Over 8 years of animal handling and performing research studies using animal models. Has had all NHEERL-required training.
Exemption 6	Technical Staff	Animal husbandry and assisting with dosing and monitoring animals	Over 6 years of animal handling and has had all NHEERL required training
Exemption 6	Technical Staff	Assist with dosing and monitoring animals	Over 18 years of animal handling and has taken all NHEERL required training
Exemption 6	Associate Principal Investigator	Assist with dosing and vaginal smears as needed when other are unavailable.	Over 35 years of animal handling and has taken all NHEERL required training.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year

2. Breeding protocols and recordkeeping

3. Methods for monitoring genetic stability

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Experimental animals will be euthanized 24 or 48 hours after dosing.

2. Describe the euthanasia techniques:

Method(s): Decapitation without anesthesia

Agent(s):

Dose (mg/kg):

Volume:

Route:

Source(s) of information used to select the above agents/methods:

2013 AVMA Guidelines on Euthanasia.

Regarding decapitation, an alternate guillotine will be readily available when animals are to be euthanized. Also, all staff that perform this method of euthanasia are highly experienced with this procedure.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing

more than 200 grams).
Decapitation without anesthesia is compliant with 2013 AVMA Guidelines for Euthanasia.

4. Describe how death is to be confirmed.
Prolonged absence of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Euthanized by Animal Care Contractor
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	08/31/2015

Submitted: 09/02/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	09/02/2015	Exemption 6	TAD	MD
		Lotus Notes Address	***Branch***	***Submitted to Branch Chief for Approval***
	by Exemption 6 Exemption 6	Exemption 6 Exemption 6 Exemption 6	NB	09/02/2015 10:10 AM

	Exemption 6 /RTP/USEPA/US	Exemption 6 RTP/USEPA/US		

ATTACHMENTS

18-09-003 PI resp.pdf

Actions

First Update notification sent: 07/27/2016
Second Update notification sent:
First 2nd Annual notification sent: 08/07/2017
Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: